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Mycoplasma fermentans-Derived High-Molecular-Weight Material Induces Interleukin-6 Release in Cultures of Murine Macrophages and Human Monocytes

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A *Mycoplasma fermentans*-derived high-molecular-weight material (MDHM) is described which causes differentiation of concanavalin A-stimulated CBA/J or C57BL/6 mouse thymocytes to cytolytic effector T cells (CTLs). The effect of MDHM was inhibited by addition of monoclonal anti-interleukin-6 (IL-6) antibody. It could also be abolished after removal of adherent cells. However, adherent cell-depleted thymocytes could still form CTLs after addition of IL-6. The action of MDHM could thus be explained by the capacity of MDHM to stimulate IL-6 release from adherent cells. MDHM was active on macrophages from CBA/J and C3H/HeJ endotoxin nonresponder mice and was also capable of stimulating IL-6 release from human monocytes. On gel chromatography, MDHM had an apparent molecular size of 1.5×10^6 daltons. Treatment with RNase and DNase had no effect on either size or biological activity. Proteinase K did not abolish activity but reduced the apparent molecular size of MDHM. MDHM production by *M. fermentans* required either coculture with eucaryotic cell lines in RPMI 1640 medium with fetal calf serum or addition of eucaryotic cell sonic extracts to this medium. The biological activity of MDHM is not identical to that of a mitogen for murine spleen cells derived from *M. arthritidis*; MDHM caused only slight proliferation in this system compared with the mitogen from *M. arthritidis*, and the latter did not elicit IL-6 release from macrophages. The results are discussed in relation to mycoplasmas as putative etiological agents for rheumatoid arthritis, since high IL-6 titers were reported for synovial fluid from patients with this disease.

Several mycoplasma-derived factors have been reported in the past that act on immune cells in various ways. A factor in conditioned media from *Mycoplasma hyorhinis*-infected cell lines was shown to suppress cytotoxic responses of T cells (24). A small basic protein, originating from *M. arthritidis*, was described by Atkin et al. as a potent T-cell mitogen (1). Material with similar activity was isolated from the same organism by Kirchner and co-workers, who described its T-cell mitogenicity (15) and potential to induce interferon-τ in leukocyte cultures (14). The activity of this factor was found to be genetically restricted to certain mouse strains (7, 11). Mitogenicity is not confined to products from *M. arthritidis* (4).

We describe here an *M. fermentans*-derived high-molecular-weight material (MDHM) that is not mitogenic for murine spleen cells but stimulates release of high titers of IL-6 from murine peritoneal macrophages and human monocytes. We found that MDHM indirectly, through IL-6, also activates thymocytes to become cytolytic. Since mycoplasmas have been implicated as an etiological agent of rheumatoid arthritis, these findings may be of clinical significance. They may explain some of the phenomena observed in chronically inflamed arthritic joints, such as excessive production of IL-6 in the synovial fluid of arthritis patients (12, 13, 29), resulting in T- and B-cell activation.

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MATERIALS AND METHODS

Mice. Male C57BL/6 mice were purchased from Charles River (Sulzfeld, Federal Republic of Germany) or Zentralinstitut für Versuchstierzucht (Hannover, Federal Republic of Germany). Female CBA/J and C3H/HeJ mice were obtained from Bomholtgaard (Ry, Denmark).

Lymphokines, antibodies, and mitogens. Human recombinant IL-6 was a generous gift of W. Fiers (Rijksuniversiteit Gent) to the Institute for Immunology in Mainz, Federal Republic of Germany. Anti-murine IL-6 antibody (28) was obtained from J. van Snick (Ludwig Institute for Cancer Research, Brussels, Belgium). Mitogen from *M. arthritidis* (MAS) was placed at our disposal by J. Homfeld and H. Kirchner (Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany). Concanavalin A (ConA) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and purified phytohemagglutinin was obtained from Wellcome Reagents Ltd. (Beckenham, England). Lipopolysaccharide (LPS) from *Salmonella typhimurium* was prepared in this laboratory by phenol-water extraction (30).

Cell lines. P815 DBA/2 mouse mastocytoma cells were used as target cells in the cytotoxicity assay. 7TD1 hybridoma cells were used for the determination of IL-6 (27). The following cell lines were used in MDHM induction experiments: U 937, a human myeloid cell line; Daudi, a human B-cell line; Molt 4, a human T-cell line; HL 60, a human myeloid cell line; and RBL-2H3, a rat basophilic leukemia cell line (8).

Cell culture. Cells were cultured at 37°C in a humidified incubator with 7.5% CO_2. 7TD1 hybridoma cells were maintained in RPMI 1640 with 5% heat-inactivated fetal calf

serum (FCS)–2 mM glutamine with the addition of 5% (vol/vol) culture supernatant from ConA-stimulated murine spleen cells as a source of IL-6. All other cell lines were kept in RPMI 1640 with 10% FCS–2 mM glutamine.

Identification of *M. fermentans*. The *M. fermentans* strain used in this study (D15-86) was isolated from a contaminated HL 60 line. It was propagated in modified Friis medium (10) consisting of Hanks balanced salt solution (10×; 50 ml), deionized water (1,230 ml), Bacto brain heart infusion (Difco Laboratories, Detroit, Mich.; 8.2 g), Bacto PPLO broth (Difco; 8.7 g), swine serum (heated at 56°C for 30 min; 150 ml), fresh yeast extract (50% [wt/vol]; 30 ml), phenol red (1% [wt/vol]; 1.25 ml), and penicillin (2,000 IU ml^{-1}). *M. fermentans* D15-86 was identified by an indirect immunofluorescence test (9).

Preparation of MDHM. MDHM was enriched from growth medium of *M. fermentans*-infected HL 60 cells as follows. Infected HL 60 cells were cultured for 4 days and then removed by low-speed centrifugation (200 × *g* for 7 min). Mycoplasmas and cell debris were spun down at 21,000 × *g* for 1 h, and MDHM was precipitated from the supernatant by 90% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in a minimum of Dulbecco phosphate-buffered saline (D-PBS) and dialyzed exhaustively against this buffer. Occasional turbidity was removed by 1 h of centrifugation at 21,000 × *g*. The MDHM preparation was routinely tested for the absence of mycoplasmas by adding samples to mycoplasma-free HL 60 cultures and staining the cells for mycoplasmas after 4 to 5 days with 2,6-diamidino-2-phenyl-indol (Boehringer GmbH, Mannheim, Federal Republic of Germany) (20).

MDHM detection by induction of cytolytic effector T cells (CTLs) in thymocyte cultures. Single-cell suspensions of thymocytes were prepared in Hanks balanced salt solution containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.4 (BSS), by gentle teasing of thymus tissue from C57BL/6 or CBA/J mice 5 to 7 weeks of age. Debris was removed by filtration through a nylon sieve. In some experiments, CBA/J thymocytes were depleted of adherent cells. To this end, 1×10^8 to 2×10^8 thymocytes were applied to a 20-ml Sephadex G10 column (Pharmacia, Uppsala, Sweden) equilibrated with BSS–5% FCS. Nonadherent cells were eluted with the same buffer and collected. Thymocytes were adjusted with RPMI 1640–5% FCS–2 mM glutamine–2.5×10^{-5} M 2-mercaptoethanol to 4×10^6 cells per ml (CBA/J cells) or 6×10^6 cells per ml (C57BL/6 cells), and ConA was added to a concentration of 8 μg/ml. Volumes (100 μl) of cell suspension were added to 100 μl of serial sample dilutions (MDHM or other mediators) in round-bottom microtiter plates (Greiner, Nürtingen, Federal Republic of Germany). Alternatively, 500 μl of cell suspension and 500 μl of sample were combined in 13-ml round-bottom tubes (Falcon tubes; Becton-Dickinson, Heidelberg, Federal Republic of Germany) when effector-to-target-cell titrations were intended. Lectin-mediated lysis was determined after a 3-day cultivation period as described previously (18). Briefly, 100 μl of supernatant was removed from each well and replaced by 100 μl of medium with 10^4 phytohemagglutinin-coated ^{51}Cr-labeled P815 cells. Alternatively, viable cells were counted in the Falcon tube cultures and adjusted to 10^6 cells per ml in RPMI 1640–10% FCS. One-hundred-microliter serial dilutions of these effector cells were mixed in round-bottom microtiter plates with 10^4 ^{51}Cr-labeled target cells. ^{51}Cr release was determined after 4 h, and percent specific release was calculated from the appropriate controls.

MDHM detection by IL-6 induction in cultures of murine peritoneal macrophages. Female CBA/J or C3H/HeJ mice 8 to 12 weeks of age were asphyxiated with CO_2. Peritoneal cells were obtained by washing the peritoneal cavity with chilled RPMI 1640–10% FCS–2 mM glutamine–2.5×10^{-5} M 2-mercaptoethanol. Cells were adjusted to 5×10^5 cells per ml and were incubated in 0.6-ml portions in 24-well tissue culture plates (Nunc, Denmark) or in 5-ml portions in 25-cm^2 tissue culture flasks for 18 h. Nonadherent cells were then removed by repeated washing with BSS. Adherent cells were cultured in RPMI 1640–1% FCS–2 mM glutamine–2.5×10^{-5} M 2-mercaptoethanol and either MDHM or phenol-extracted LPS from *S. typhimurium* as indicated. IL-6 was determined in the culture medium after 24 h unless otherwise indicated.

MDHM-mediated IL-6 production by human monocytes. Blood from healthy donors was diluted with an equal volume of D-PBS, and mononuclear cells were isolated on a cushion of Lymphoprep (Nyegaard, Oslo, Norway) according to the instructions of the supplier. The cells were freed of thrombocytes by repeated low-speed sedimentations (200 × *g* for 7 min). Mononuclear cells were adjusted to 1.5×10^6 cells per ml in RPMI 1640–10% FCS. Portions (7.5 ml) of the cell suspension were incubated in 18-cm^2 petri dishes. Nonadherent cells were removed after 2 h by careful washing with BSS. Adherent cells were cultured for the indicated times in RPMI 1640–2% FCS in the presence of MDHM or other stimuli.

IL-6 assay. IL-6 was tested in a modified hybridoma growth assay in 96-well microtiter plates (27). Briefly, 10^4 7TD1 cells were mixed with serial dilutions of IL-6 samples in a final volume of 100 μl in RPMI 1640–5% FCS–2 mM glutamine–2.5×10^{-5} M mercaptoethanol. Proliferation was measured after 3 days by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) reduction test (17). MTT solution (10 μl) (5 mg of MTT per ml of D-PBS) was added to each well, and cultivation was continued for 4 h. Subsequently, 120 μl of a 5% formic acid solution in isopropanol was added to each well. The blue formazan dye was dissolved by sonication and measured in a microelisa reader (Intermed NJ 2000; Nunc) at 577 nm, with a reference adsorption at 656 nm. The 7TD1 line did not react to MDHM, the cytokines IL-1, IL2-, IL-3, IL-4, IL-5, and IL-7, or the colony-stimulating factors M-CSF, G-CSF, or GM-CSF (E. Schmitt, unpublished).

Infection of cell lines with mycoplasmas. Ten-milliliter cultures of U 937, Daudi, and Molt 4 cells were seeded at 2×10^5 cells per ml, P815 and RBL-2H3 cultures were seeded at 5×10^4/ml and 5×10^3/ml, respectively, and all were infected by addition of 1% (vol/vol) medium containing about 3×10^8 CFU of *M. fermentans* D15-86. Cells were harvested 3 days later, checked for mycoplasma infection with 2,6-diamidino-2-phenyl-indol stain, and transferred into new medium at the cell concentrations described above. After another 4-day growth period, eucaryotic cells were removed by low-speed centrifugation (200 × *g* for 7 min) and the medium was freed of mycoplasmas by centrifugation for 45 min at 21,000 × *g*. MDHM activity was tested in the supernatants. In the experiments with P815 and RBL-2H3 cells, the second culture period was omitted.

Mycoplasma growth and MDHM production in RPMI 1640 medium substituted with HL60 sonicates. HL 60 cells grown to 10^6 cells per ml in RPMI 1640–10% FCS–2 mM glutamine were sonicated six times for 30 s each (60 W, Sonifier B 12; Branson Sonic Power Company, Danbury, Conn.). Debris was sedimented by a 45-min centrifugation at 21,000 × *g*,

and the supernatant was passed through a filter (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.). Fresh glutamine (2 mM) was added, and the filtrate was inoculated with *M. fermentans*. After a 6-day cultivation period, the medium was freed of mycoplasmas by centrifugation for 45 min at 21,000 × *g*, and possibly remaining mycoplasmas were inactivated by heat treatment (1 h, 57°C). Heat-stable MDHM activity was determined by the IL-6 induction assay.

Molecular characterization of MDHM. (i) Ultracentrifugation. MDHM was centrifuged for 1 h at 105,000 × *g*. MDHM activity was tested in the supernatant, the suspended pellet, and the untreated control preparation.

(ii) Nuclease treatment. To an MDHM preparation in RPMI 1640, RNase and DNase I (both from Boehringer) were added at final concentrations of 80 μg/ml (RNase; 40 Kunitz U/mg) and 100 μg/ml (DNase; 2,000 U/mg). After a 4-h incubation at 37°C, a 1.35-ml sample was subjected to gel filtration on a Sephacryl S 300 column (1.6 by 34.5 cm) (Pharmacia, Freiburg, Federal Republic of Germany). The elution buffer was D-PBS, and the flow rate was 10 ml/h. MDHM activity in the eluate was tested by the CTL induction assay.

(iii) Protease treatment. MDHM was separated from bulk protein by gel filtration on a Sephacryl S 300 column as described above. Fractions containing the main MDHM activity were pooled from several runs and concentrated by ultrafiltration (Amicon PM 10; Amicon Corp., Lexington, Mass.). To 2.2 ml of such a MDHM preparation containing 2.29 mg of protein, 0.46 mg of proteinase K (27 milli-Anson U/mg; Merck-Schuchardt, Darmstadt, Federal Republic of Germany) was added. The reaction was stopped after a 1-h incubation at 37°C by addition of 5% (vol/vol) FCS, and the sample was immediately applied to a Sephacryl S 300 column as described above. MDHM activity in the eluate was tested by the CTL induction assay.

Spleen cell proliferation assay. Splenocytes from CBA/J mice (10 to 12 weeks of age) were prepared as described for thymocytes. The cell concentration was adjusted to 6×10^6 cells per ml in RPMI 1640–5% FCS–2 mM glutamine. One hundred microliters of cell suspension was added to 100-μl serial dilutions of potential mitogens in round-bottom microtiter plates. Cells were cultured for 3 days. For the last 7 h, 1 μCi of [*methyl*-^{3}H]thymidine per well (specific activity, 0.25 mCi/μmol; Amersham-Buchler, Braunschweig, Federal Republic of Germany) was added in 20-μl volumes to the cultures. Acid-insoluble material was precipitated on glass fiber filters with a multiple automatic sample harvester, and radioactivity was determined in a liquid scintillation counter.

RESULTS

Discovery of MDHM as a helper factor for thymocyte differentiation. MDHM was first discovered as an activity in the culture medium of *M. fermentans*-infected HL 60 cells that allowed the differentiation of murine thymocytes to CTLs. MDHM-dependent CTL development, measured by a lectin-dependent ^{51}Cr release assay, showed a distinct dose response (Fig. 1). MDHM did not act on thymocytes which had been depleted of adherent cells by a passage over Sephadex G 10 (Fig. 2), suggesting that its effect might be indirect, possibly involving factors released from the adherent-cell population. Two findings speak in favor of IL-6 as a mediator in this system. (i) CTL response could be reconstituted by addition of murine or human recombinant IL-6 to adherent cell-depleted thymocytes (Fig. 2), and cytolytic activity increased with the IL-6 activity over a wide range (Fig. 3). (ii) In addition, MDHM-mediated CTL differentiation was inhibited by a monoclonal anti-IL-6 antibody (Table 1). This inhibition could be overcome by IL-2, which indicates that the antibody preparation (ascitic fluid) was not unspecifically toxic at the dilution used (Table 1).

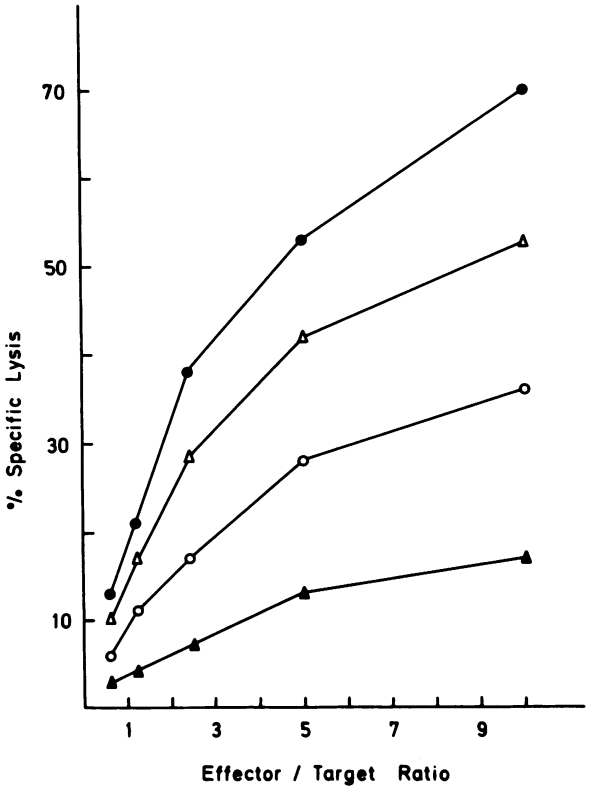

FIG. 1. MDHM-mediated formation of CTLs from thymocytes. C57BL/6 thymocytes (3×10^6/ml) were cultured in the presence of ConA and various concentrations of MDHM. Viable cells were harvested after 3 days. PHA-mediated lysis of 10^4 ^{51}Cr-labeled P815 target cells was determined in a 4-h ^{51}Cr release assay at the effector/target ratios indicated. Symbols: ▲, ConA only; ○, 0.25% (vol/vol) MDHM; △, 0.75% (vol/vol) MDHM; ●, 1.5% (vol/vol) MDHM. Data are means of duplicate samples and represent one of four independent experiments.

MDHM induces IL-6 release. In order to clarify whether the role of MDHM was to provide IL-6 from adherent cells as one obligatory step in CTL development (19, 23, 25), we next tested the capacity of MDHM to cause IL-6 release from murine peritoneal macrophages and human peripheral blood monocytes. MDHM was able to rapidly stimulate IL-6 production (Table 2). IL-6 release from MDHM-stimulated human monocytes was equally fast and led to comparable IL-6 release (1,280 U/ml after 24 h [medium control, 256 U/ml]). A parallel, LPS-stimulated culture produced 1,280 U of IL-6 per ml. MDHM-mediated IL-6 release was concentration dependent (Fig. 4). This direct effect of MDHM on the release of IL-6 from macrophages was a more sensitive assay for MDHM than the cytotoxicity test which, however, has the advantage that it covers a wider concentration range of MDHM (compare Fig. 4 with Fig. 1). Accordingly, depending on the requirement, either MDHM-dependent differentiation of thymocytes to CTLs or MDHM-mediated IL-6 production by peritoneal macrophages was used to measure this activity.

MDHM, a product of *M. fermentans*. When we first de-

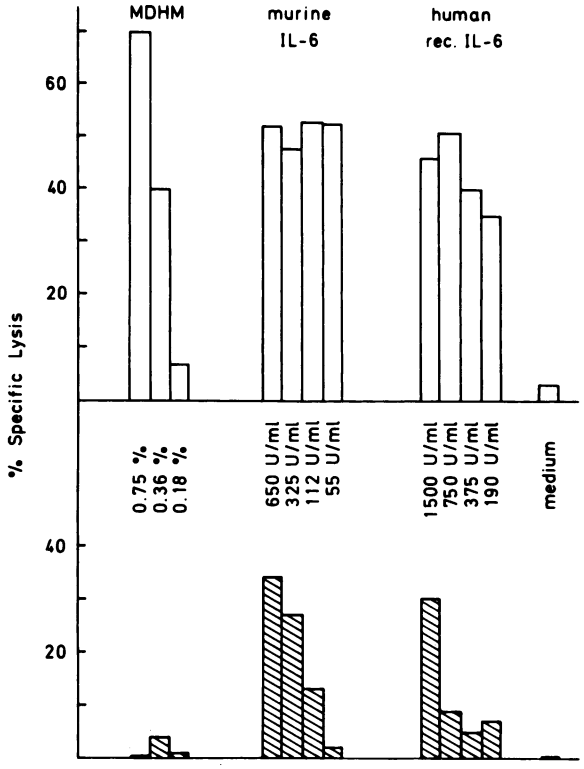

FIG. 2. Effects of MDHM and IL-6 on CTL formation from untreated and adherent cell-depleted thymocytes. CBA/J thymocytes were depleted of adherent cells by passage over a G10 Sephadex column. A total of 4×10^5 of untreated (top) or adherent cell-depleted (bottom) thymocytes per 0.2 ml were cultured in the presence of ConA (medium control) and various concentrations of MDHM, murine IL-6, or human recombinant (rec.) IL-6. Development of CTLs was determined after 3 days by PHA-mediated lysis of 10^4 ^{51}Cr-labeled P815 target cells. Data are means of duplicate samples and represent one of four experiments.

tected MDHM activity in the culture medium of the mycoplasma-infected human cell line HL 60, it was unclear whether this material was a product of the mycoplasmas themselves or of these particular eucaryotic host cells,

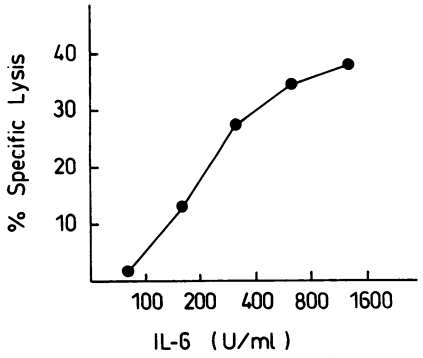

FIG. 3. Concentration dependency of IL-6-mediated CTL formation from thymocytes. Adherent cell-depleted CBA/J thymocytes were cultured at 4×10^5 cells per 0.2 ml in the presence of ConA and the indicated amounts of murine IL-6. Specific lysis was determined after 3 days as described for Fig. 2. Specific lysis of control cultures (ConA only) was 0. Data are means of duplicate samples and represent one of four experiments.

TABLE 1. Role of IL-6 in the MDHM-mediated generation of CTLs in cultures of ConA-stimulated thymocytes

Stimulus (amt)	Addition	CTL activity[a] (% specific lysis)
MDHM (1.5% [vol/vol])	None	33
MDHM (1.5% [vol/vol])	α-IL-6 MAb[b]	7
Medium	None	8
MDHM (1.5% [vol/vol])	α-IL-6 MAb plus IL-2 (30 U/ml)	40

[a] Thymocytes were cultured in the presence of 4 μg of ConA per ml and, where indicated, other additions, which were added at the beginning of the culture period. CTLs were harvested after 3 days and PHA-mediated lysis of ^{51}Cr-labeled P815 target cells was determined as an effector-to-target ratio of 10/1. Data are means of duplicate determinations. The experiment was performed twice.

[b] MAb, Monoclonal antibody; (final dilution, 1:10).

possibly as a result of stimulation by the mycoplasmas, since uninfected HL 60 cells did not produce MDHM. The mycoplasmas were isolated from the culture medium and serologically identified as *M. fermentans*. Attempts to detect MDHM in modified Friis medium in which *M. fermentans* had been cultivated failed, because preparations from this medium proved to be toxic to the thymocytes. On the other hand, *M. fermentans* failed to grow and produce MDHM in RPMI 1640 medium containing all additions required to grow animal cells. We therefore infected several eucaryotic cell lines with *M. fermentans* from HL 60 cultures. MDHM could be detected in the culture medium of every infected cell line, whereas none of these lines produced MDHM before infection (Table 3). *M. fermentans* cultured for several growth cycles in modified Friis medium could infect and induce production of MDHM in the cell lines equally well. Since it was improbable that all cell lines tested, regardless of their species origin and ontological background, could produce the same biological activity, it became more likely that MDHM was a product of *M. fermentans* which, at least when grown in RPMI 1640 medium, was only formed under conditions of cocultivation with animal cells. We therefore tested whether RPMI 1640 medium containing a sonic extract from noninfected HL 60 cells could serve as a medium for growth of *M. fermentans* and production of MDHM. The

TABLE 2. Kinetics of MDHM-mediated IL-6 release from CBA/J mouse peritoneal macrophages

Incubation time (h)	IL-6 activity (U/ml)[a]	
	MDHM stimulated[b]	Medium controls
0.25	4	<4
0.5	8–16	4
1.0	64–128	4
2.0	320–640	4
4.0	640–1,280	16
8.0	640	8
10.0	640–1,280	8–16
13.5	1,280	8–16
25.0	800–1,600	16
30.0	800–1,600	8–16

[a] IL-6 in the culture medium was determined in duplicate serial dilutions by the hybridoma growth assay; 1 U of IL-6 corresponds to the dilution giving half-maximal growth. The data represent the range of these dilutions. The experiment was performed twice.

[b] MDHM (2% [vol/vol]) was added at zero time to 0.6-ml cultures of resident macrophages.

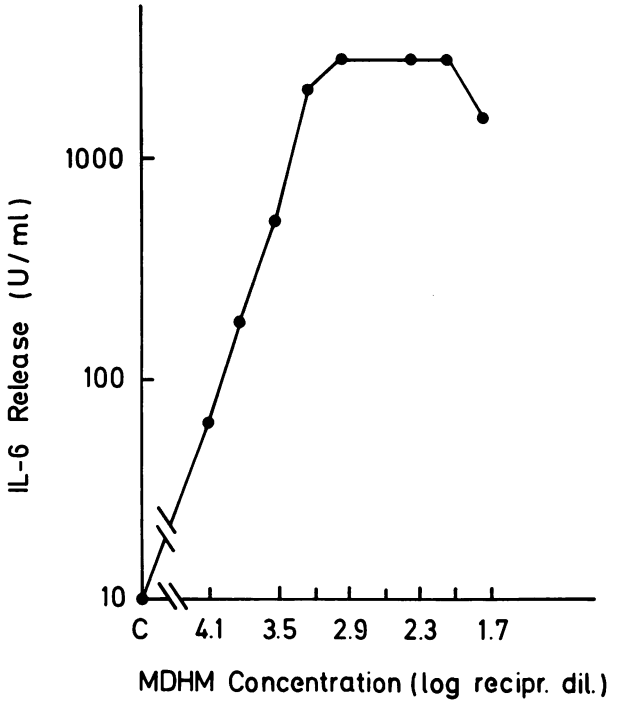

FIG. 4. Concentration dependency of MDHM-mediated IL-6 release from peritoneal macrophages. Resident peritoneal macrophages from CBA/J mice (3×10^5 per 0.6 ml) were cultured for 24 h in the presence of the indicated concentrations of MDHM. IL-6 in the culture medium was determined in serial dilutions by the hybridoma growth assay. C indicates IL-6 release in medium controls. The data are means of duplicate IL-6 determinations.

data in Table 4 (experiment 1) illustrate that *M. fermentans*, when cultured in this cell homogenate, produced MDHM capable of inducing high IL-6 titers.

Molecular characterization of MDHM. When a preparation of MDHM was centrifuged for 1 h at 105,000 × *g*, most MDHM activity remained in the supernatant, although some activity was found in the microsomal pellet. Gel filtration on a Sephacryl S 300 column identified MDHM as a high-molecular-weight material, eluting very close to the void volume of this molecular sieve, which has a fractionation range of 10^4 to 1.5×10^6 daltons (Fig. 5). Incubation of

TABLE 3. MDHM production in culture media of *M. fermentans*-infected cell lines

Cell line (origin)	MDHM activity (% specific lysis)[a]	
	M. fermentans infected	Before *M. fermentans* infection
U937 (human; myeloid)	51	1
Daudi (human; B cell)	54	0
Molt 4 (human; T cell)	16	0
P815 (mouse; mastocytoma)	9	1
RBL-2H3 (rat; mast cell)	10	0

[a] MDHM activity was determined by the capacity to raise CTLs in ConA-stimulated C57BL/6 mouse thymocyte cultures. Five percent (vol/vol) of each culture medium, freed of myocplasmas by high-speed centrifugation, was added to the thymocyte test cultures. Control thymocyte cultures with ConA only had 0 to 3% specific lysis; positive controls (1.25% [vol/vol] MDHM added) had 18 to 21% specific lysis. Data are means of duplicate determinations. The experiments were conducted at least twice.

TABLE 4. IL-6 release from murine peritoneal macrophages in response to various stimuli

Expt no.	Macrophage source	Sample added	IL-6 activity[a] (U/ml)
1[b]	CBA/J mouse	MDHM in *M. fermentans*-infected, HL 60 lysate-enriched RPMI 1640 medium (25% [vol/vol])	2,080
	CBA/J mouse	HL60 lysate-enriched RPMI 1640 before infection (25% [vol/vol])	32
	CBA/J mouse	Medium control	64
	CBA/J mouse	MDHM (2.5% [vol/vol])	1,600
2[b]	CBA/J mouse	MAS (0.1% [vol/vol])	32
	CBA/J mouse	Medium control	64
	CBA/J mouse	MDHM (2.5% [vol/vol])	1,600
3[c]	CBA/J mouse	MDHM (0.2% [vol/vol])	2,500
	CBA/J mouse	LPS (1 μg/ml)	2,500
	CBA/J mouse	Medium control	16
4[c]	C3H/HeJ mouse	MDHM (0.2% [vol/vol])	256
	C3H/HeJ mouse	LPS (1 μg/ml)	<4
	C3H/HeJ mouse	Medium control	<4

[a] IL-6 activity was determined by the hybridoma growth test as described for Table 2.

[b] Cultures were done in 25-cm^2 tissue culture flasks. HL 60 lysate-enriched medium was RPMI 1640 medium substituted with sonic extracts from HL 60 cells. Stimulating materials were freed of mycoplasmas by high-speed centrifugation and added at zero time. IL-6 activity was determined after 48 h.

[c] Cultures were done in 0.6-ml volumes in multiwell tissue culture plates. Stimulants were added at zero time. IL-6 activity was determined after 24 h.

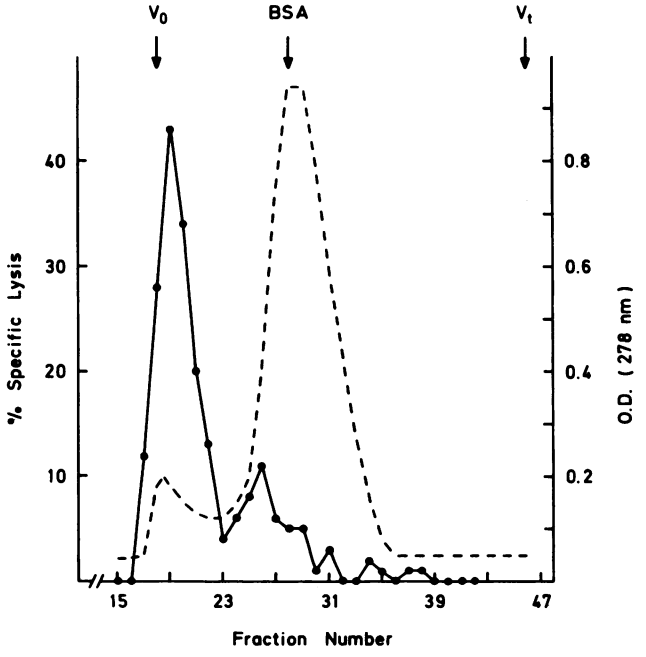

FIG. 5. Sephacryl S 300 gel filtration of MDHM. C57BL/6 thymocytes (6×10^5 cells per 0.2 ml) were cultured with ConA and 25% (vol/vol) of each fraction. Specific lysis of ^{51}Cr-labeled P 815 target cells was determined as described for Fig. 2. ——, ^{51}Cr release; - - - - -, optical density at 278 nm. Data are means of duplicate determinations and represent one of four experiments. V_0, Exclusion volume; V_t, total volume.

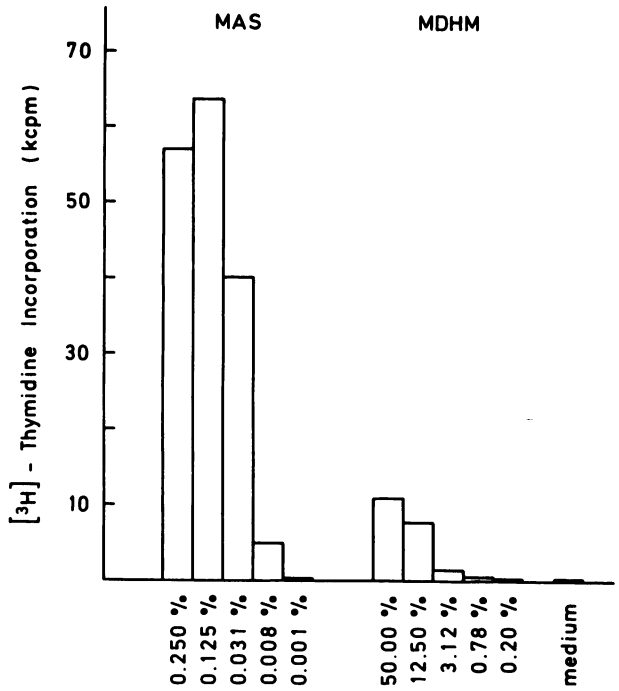

FIG. 6. Proliferation of CBA/J spleen cells in response to MDHM and MAS. Splenocytes (6×10^5 cells per 0.2 ml) were cultured for 3 days in the presence of various concentrations of MDHM and MAS. Proliferation was determined by [^{3}H]thymidine incorporation during the last 7 h of incubation. The data are means of duplicate determinations and represent one of two experiments.

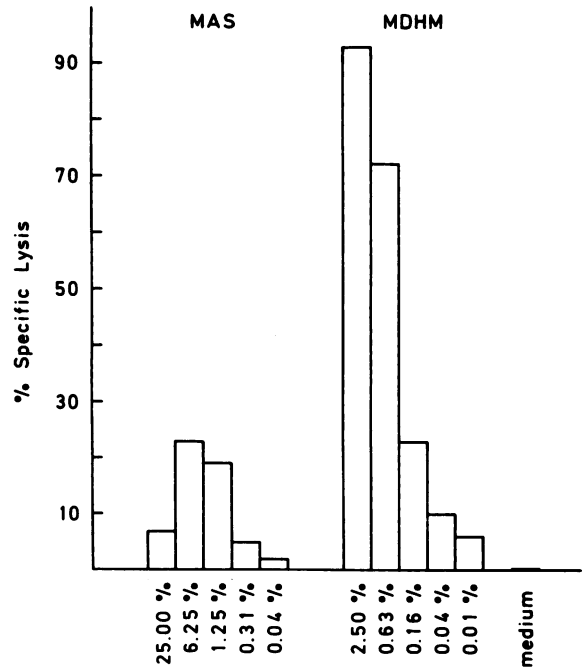

FIG. 7. Formation of CTLs from C57BL/6 thymocytes in response to MDHM and MAS. ConA-stimulated thymocytes (6×10^5 cells per 0.2 ml) were cultured in the presence of various concentrations of MDHM and MAS. Specific lysis of ^{51}Cr-labeled P 815 target cells was determined as described for Fig. 2. Data are means of duplicate determinations and represent one of two experiments.

MDHM with RNase and DNase before gel filtration did not change this elution pattern. Prior incubation of MDHM with proteinase K, however, led to disappearance of the peak near the exclusion volume, causing MDHM activity to spread over a broad elution range (not shown).

Comparison of MDHM with a soluble mitogen from *M. arthritidis*. MAS, a substance with activity from *M. arthritidis*, is known as a potent mitogen for spleen T cells of various mouse strains (1, 15). We compared the effects of MAS and MDHM in three assay systems by testing stimulation of thymidine uptake in CBA/J spleen cell cultures, induction of CTLs in C57/BL6 thymocyte cultures, and generation of IL-6 in CBA/J macrophage cultures. Whereas MAS stimulated an almost 200-fold proliferation of spleen cells over that in control cultures, MDHM caused very little response (Fig. 6). The influence of the two materials on the development of thymocytes to CTLs is shown in Fig. 7. In contrast to MDHM, even highly purified MAS had only marginal activity in this assay. Moreover, MAS, when used at concentrations which caused maximal proliferation in the spleen assay, did not induce IL-6 production in macrophage cultures (Table 4, experiment 2). These experiments strongly suggest that MDHM and MAS are different biological materials.

Macrophages from C3H/HeJ LPS nonresponder mice respond to MDHM. The capacity of MDHM to stimulate IL-6 release in macrophage cultures was reminiscent of LPS effects (26). It was therefore important to study the influence of MDHM on macrophages from genetically LPS nonresponder mice (21, 22) to exclude a contamination of our preparations with LPS. MDHM led to IL-6 release in CBA/J and C3H/HeJ macrophage cultures, whereas LPS acted only on CBA/J macrophages (Table 4, experiments 3 and 4).

DISCUSSION

Studies in our laboratory which were originally undertaken in order to search for cytokines which caused differentiation of CTLs from thymocytes led to the discovery of a biological activity that had the desired effect but which was only generated in the presence of live *M. fermentans*. According to the data presented here, this material, which we have tentatively named MDHM, acts via liberation of IL-6 by accessory cells and is not identical to MAS from *M. arthritidis*, nor is its activity due to bacterial endotoxin. These points, as well as possible implications of our findings for inflammatory processes such as occur in rheumatoid arthritis, will be discussed in detail below.

Active MDHM was initially detected in culture media of several cell lines infected with *M. fermentans*. None of these culture media contained CTL-inducing activity before infection. Detection of MDHM in conventional mycoplasma growth media infected with *M. fermentans* was not feasible for two reasons. (i) These media, even at high dilutions, appeared to be toxic to the thymocyte cultures. No elaborate attempts were made to separate this toxic material from MDHM because (ii) the horse and swine sera used in this medium were obtained from a slaughterhouse and are very likely to be contaminated with endotoxin, which would interfere with the assay, giving false-positive results. In later experiments, however, we succeeded in producing MDHM by culturing *M. fermentans* in a cell culture medium which was compatible with our assay systems by use of a sonic extract of HL 60 or P815 cells as a substitute for viable host cells. This rules out host cells as the primary source of MDHM and makes it more likely that components from the host cells are simply required as nutrients for *M. fermentans*. However, a possible modification of host cell material by *M. fermentans* to yield MDHM has still to be considered.

MDHM was shown here to induce IL-6 release in cultures of human peripheral blood monocytes and murine peritoneal macrophages. Our own unpublished preliminary data suggest that TNF and arachidonic acid metabolites are also induced. Two facts point to IL-6, however, as a key cytokine that is required to drive CTL generation in the thymocyte system. (i) MDHM-stimulated CTL formation was inhibited by a specific anti-IL-6 antibody (Table 1), and (ii) thymocytes, unreactive to MDHM after the removal of adherent accessory cells, were still able to form CTLs when reconstituted by IL-6 (Fig. 2).

MDHM remained in the supernatant after ultracentrifugation. According to our gel chromatography experiments, it is a high-molecular-weight complex (>1.5 × 10^6 daltons). In contrast to the *M. arthritidis*-derived mitogen MAS, a small basic protein which forms noncovalent complexes with nucleic acids (1), the apparent molecular size of MDHM was not changed by DNase or RNase treatment. However, incubation of MDHM with proteinase K, although it did not destroy MDHM activity, led to a retardation of MDHM on the molecular sieve gel and a broadening of the activity profile. This suggests that MDHM might be a smaller nonprotein molecule binding to a protein-containing carrier. There are other differences distinguishing the *M. fermentans* product MDHM from the *M. arthritidis*-derived MAS, e.g., whereas MAS is a highly effective mitogen for CBA/J spleen cells (6, 15), MDHM caused only a slight response in the proliferation assay. MDHM very efficiently induced cytolytic activity in CBA/J or C57/BL6 thymocytes, while highly purified MAS showed no more than marginal effects in this system. This is explained by our finding that MDHM caused IL-6 production by CBA/J peritoneal macrophages, whereas MAS was inactive in this assay, even at concentrations inducing maximal proliferation of spleen cells. LPS from gram-negative bacteria shows biological effects similar to those of MDHM. We could exclude an LPS contamination of our material by showing that MDHM induced IL-6 production in peritoneal macrophages of C3H/HeJ LPS-nonresponder mice, in which LPS was inactive.

Our findings that an *M. fermentans* product, like endotoxin, induces IL-6 release from monocytes and/or macrophages are of general interest in the context of inflammatory processes, in particular in the arthritic joint. Thus, high concentrations of IL-6 were recently reported in the synovial fluid of patients with rheumatoid arthritis (12, 13, 29), and the suggestion was made that the infiltration of the synovium by lymphocytes and plasma cells in affected joints may be due to IL-6 (12). Furthermore, IL-6 has pleiotropic effects on B and T lymphocytes (32) as well as on phagocytes (3). In particular, it serves as a helper factor in the in vitro induction of human and murine cytolytic T cells (19, 23). It is interesting to recall that along with other mycoplasmas, *M. fermentans* has been discussed as a possible cause for rheumatoid arthritis in humans (16, 31), although isolation and characterization of these microorganisms from the joints of patients has proved difficult, and the results have been conflicting (2, 5).

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